Real-Time Binding Kinetic Analyses of the Interaction of the Dietary Stain Orange II with Dentin Matrix

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Abstract

Objectives: Dietary stains can be adsorbed into the dentin of teeth. Using Orange II as a model dietary stain, this study investigated the strength of its interaction with the mineral and protein components of dentin matrix and how hydrogen peroxide (H_2O_2) treatment influences this interaction.

Methods: Dentin slices were prepared from human teeth and were either deproteinized (5.6% sodium hypochlorite, 12 days), demineralised (0.5M EDTA, 3 days) or left as intact control samples. Samples were stained with Orange II for 1-168h, during which staining intensity was quantified by image analysis. Similarly, uptake of stain by deproteinized / demineralized samples treated with 10 or 30% H_2O_2 was investigated. Using surface plasmon resonance technology, real-time binding kinetics were determined assessing the interaction of orange II with the dentin matrix protein constituents, collagen type I, biglycan, decorin, dentin sialoprotein and osteopontin.

Results: Deproteinization of dentin matrix reduced the uptake of the orange II compared to the intact control. Conversely, demineralization of dentin samples increased the uptake of the dye. Treatment of samples for 48h with H_2O_2 reduced subsequent uptake of the orange II. Real-time kinetic analysis indicated moderate strength of binding for Orange II with collagen type I, weak binding with decorin and biglycan and negligible binding with dentine sialoprotein and osteopontin.

Conclusion: These results indicate a predominant role for collagen type I, which accounts for 90% of the organic protein matrix of teeth, for attracting dietary stains. Binding analyses indicate that the interaction is highly dissociable, and further binding is reduced following H_2O_2 treatment.

Clinical significance

This study provides new information regarding adsorption of dietary stains into tooth dentin, suggesting that they are attracted and moderately bound to the collagen type I matrix. This study also contributes valuable information for discussion for considering the effect of H_2O_2 on bleaching teeth and its influence on subsequent uptake of dietary stains following whitening treatments.

1. Introduction

Teeth inherently uptake small dietary components which contain chromogenic organic compounds which can lead to tooth discolouration [1,2]. Such extrinsic staining is due to the porosity of the enamel, which the chromogens can penetrate and be taken up by the dentin [2]. Clinically, the natural tooth colour can be restored by several methods that focus on external bleaching of the teeth. Hydrogen peroxide (H_2O_2) is the most common agent used in tooth whitening and can be applied in different concentrations based on its intended method of application and use. Consequently, treatment formulations intended for use in the dental clinic have higher H_2O_2 (~35%) concentration and have shorter application period, whilst products for use over-the-counter, which have lower H_2O_2 concentration (2-10%) and longer application period [3-5].

How externally presented chromogenic substances interact with the tooth matrix is still not fully elucidated. Proposed mechanisms suggest that the attraction of chromogens to the tooth surface could be due to several types of attractive forces that include electrostatic van der Waals and hydration forces [1]. Although it is not fully established what part of the tooth matrix is responsible for staining, the dentin matrix is highly likely to play a key role in defining the colour of the tooth [6]. Natural micro-faults within the enamel layer are suitable to allow small dietary chromogens to enter and interact with the underlying dentin matrix [7]. Whilst it is accepted that tooth enamel could contain very small levels protein components residual from the processing of the enamel matrix [8,9], mature enamel is composed of more than 98% hydroxyapatite. In contrast, dentin has a significantly higher organic content at 30% of which consists 90% collagen and 10% non-collagenous proteins (NCPs) (reviewed by [10]). Collagen type I has a triple helix structure of two alpha 1 and one alpha 2 chains [10], which are cross linked by prolyl hydroxylase enzyme to form the collagen fibrils [11]. Within

dentin, collagen fibrilogenesis and subsequent regulation of hydroxyapatite formation associated with the mineralisation of the collagen fibrils is orchestrated by several NCPs which are highly negatively charged, including osteonectin, osteocalcin, dentin phosphoprotein, dentin sialoprotein, decorin and biglycan [10-13]. Due to these negative charges such proteins have been speculated to be involved in the attraction of dietary chromogens into the dentine matrix, causing tooth staining.

In order for H_2O_2 to bleach the tooth structure, the H_2O_2 needs to penetrate the tooth surface to reach deep to the staining site in the tooth dentin. Several studies have indicated an ability for H_2O_2 to be able to pass through the highly mineralised enamel tissue, through the porous tubular structure of the dentin and into the pulp chamber although mechanisms of passage are unclear [14-17]. Additionally, the penetration rate will vary depending on the concentration and time of H_2O_2 application, and other minor defects to the tooth structure, especially if it involves a restored tooth [17]. H_2O_2 is proposed to remove stain via the production of short-lived free radicals such as hydroxyl radical (OH^-) [18,19] which acts as strong oxidising agents that breaks carbon double bonds often found in the colour staining chromophore, such as heteroatoms, carbonyl, or phenyl rings [19]. However, as highly chemically unstable molecules, these peroxide derived free radicals are able to react indiscriminately with other organic molecules, including proteins, lipids, carbohydrates and nucleic acids (reviewed in [20]). Indeed, studies have also suggested that H_2O_2 also bleach the tooth by oxidation of the organic matrices of the tooth, thereby releasing chromogen interaction with the protein matrix [21]. Continued degradation of the tooth structure, due either to the production of the free radicals degrading the dentin matrix or due to the acidic nature of H_2O_2 causing erosion, could lead to thinning of the dentin and widening of the dentinal tubules. This can produce medical implications such as tooth sensitivity and could ultimately lead to pulp damage as H_2O_2 diffuses through enamel and dentin [15,22,23].

There are thus increasing concerns regarding safety levels for the use of H_2O_2 in tooth whitening products. In order to identify effective un-harmful treatments for tooth staining, a better understanding is required of how the biological properties of dentin structure influences inherent tooth colour. Against this background the present study investigates the physiochemical nature of the interaction of staining chromophores with the protein components of dentin and the influence of H_2O_2 on this interaction. Due to the complex nature of dietary stain we have selected to study one potential chromogen, Orange II sodium salts. This chromogen has a small molecular weight that can penetrate the tooth's structure, and contains a carbon phenol ring structure that contains H_2O_2 susceptible double carbon bonds [24] and similar to phenol chromogens of dietary sources such as tea and coffee that cause extrinsic discoloration of teeth [6,24]. Our study first identifies the attraction of Orange II predominantly with dentin proteins in situ within the tooth and assesses the effect of re-staining following treatment with H_2O_2. Studies then continue to use surface plasmon resonance technology to investigate the real-time biomolecular interactions of Orange II with collagen type I and non-collagenous dentin proteins, extracted from the dentin matrix.

2. Materials and Methods

2.1. Teeth Preparation

Twenty extracted human teeth were collected from the Cardiff University Tooth Bank with patient consent and ethical approval by the South East Wales Research Ethics Committee of the National Research Ethics Service (NRES), UK (reference number: 12/WA/0289). The outer surface of the teeth were sterilised with 70% ethanol and then all enamel, cementum, caries, restoration and tooth discolouration (i.e. any abnormal tooth colour or translucency) where removed using dental burs with slow speed handpiece (Kavo EWL K9). Longitudinal sections of dentin of 100µm thickness were prepared using an Isomet bone saw (Buehler, USA).

2.2. Sample Treatments and Colour Evaluation

Fifteen longitudinal dentin sections, prepared from five teeth, were randomly divided into 3 groups; deproteinized, demineralised and untreated control groups. Each section was placed in one well of a 6-well plate and treated with either deproteinising solution (sodium hypochlorite, 5.6%, Sigma-Aldrich, Poole, UK) for 12 days or demineralising solution (0.5M EDTA, pH 8, Sigma- Aldrich) for 3 days. Samples in the intact control group were incubated in phosphate buffered saline (PBS) for 12 days. All groups were stained with Orange II sodium salts dye (0.15mM), during which the uptake of the Orange II into the dentin slices was digitally quantified at 0, 1, 6, 24, 48, 168h as follows. Images of the whole dentine section were taken using a digital camera (Panasonic, DMC-G1) attached to a stereo microscope (Zeiss, stemi 2000) at low magnification (x1.25) under cold light (Labophot-2, Nikon Digital, Japan) to achieve standard conditions. The intensity of the dye uptake was calculated after converting the digital data from the colour images to 8-bit black and white image and quantifying the grayscale measurement using ImageJ® Software, (http://rsb.info.nih.gov/ij/). Within the programme, the grayscale value of intensity was measured and represented as a value were zero is taken to be black, and 255 is taken to be white. Values were then converted to a % greyscale of increasing staining intensity of Orange II stain. Means and standard deviations were calculated (n=3) and analysed using one-way analysis of variance (ANOVA), with Tukey post-test for multiple comparison, using GraphPad InStat3 (GraphPad Software, La Jolla, USA) to determine statistically relevant differences between the deproteinized, demineralised and control groups. A value of $p<0.05$ was considered significant.

After staining, teeth were treated with 10% or 30% H_2O_2 and colour loss was similarly monitored 0, 1, 3, 6, 24, 48h using digitised image analysis described above. Teeth

were then re-stained with Orange II sodium salts and re-incorporation of the stain was monitored at 1, 24 and 168h, as above.

2.3. Effects of H_2O_2 on Collagen Type I

Purified collagen type I (from human placenta, Sigma- Aldrich) was dissolved in 0.5M acetic acid at 1mg/ml and then mixed with either 25%, 12.5%, 6% and 3% H_2O_2 (final concentration) for 1, 12 or 24h at room temperature. Samples were mixed with equal volume of sample buffer (0.062M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-â-mercapthoethanol, 0.002% bromophenol blue; final concentration) and 30µL samples were separated by SDS-PAGE using 4-15% mini Protein TGX Precast gels (Bio-Rad, Hemel Hempstead, UK), 0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3 running buffer, 200v for 40 min. Evidence of degradation was visualised using a silver stain plus kit (Bio-Rad) following manufacturer's instructions. Gels were scanned using a Gel-Doc^{TM} EZ Imager (Bio-Rad).

2.4. Dentin Matrix Protein Extraction

The dentine matrix prepared from fifteen teeth (as described above) was powdered, using a percussion mill (SPEX CertiPrep, USA) cooled with liquid nitrogen. Dentin matrix proteins were extracted from powdered dentin into 10% EDTA (pH 7.2) containing protease inhibitors (0.1mM n-ethylmaleimide, 0.5mM Iodoacetic acid and 0.5mM Benzamidine, Sigma- Aldrich) as previously described [25]. Extraction was performed for 6 days at 4^{o}C with constant agitation, with the EDTA solution changed and retained daily. Retained dentine matrix extracts were pooled over the 6-day period, transferred to dialysis tubing (Scientific Laboratory Supplies, Nottingham, UK) and dialyzed exhaustively for 10 days against

repeated changes of deionized distilled water. Dialyzed extracts were lyophilized and stored at –20^{o}C prior to use.

2.5. Immunoprecipitation of Biglycan, Decorin, Osteopontin and Dentin Sialoprotein from Dentin Matrix Protein Extract

Immunoprecipitation of dentin associated proteins were performed using the Pierce® Crosslink Immunoprecipitation Kit (Thermo Fisher scientific, Loughborough, UK),. Briefly, 20μg antibodies against the target proteins of biglycan (PR8A4) and decorin (70.6; both a kind gift from Prof. Bruce Caterson, Cardiff University), osteopontin (OPN, sc-10593, Santa Cruz Biotechnology Inc, Texas, USA) and dentin sialoprotein (DSP, sc-33586; Santa Cruz Biotechnology Inc) were cross-linked to Protein A/G Plus Agarose contained within a spin column supplied, according to manufacturer's instructions. Lyophilized dentin matrix protein extract was reconstituted in lysis buffer (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP-40, 5% glycerol; pH 7.4, supplied within the kit) to a concentration of 2mg/mL. The dentin matrix protein extract was added to the antibody-cross-linked resin in the column which was closed-off and incubated with gentle shaking overnight at 4°C. After incubation, the column was centrifuged, washed with lysis buffer to remove unbound material and washed with conditioning buffer prior to incubation with elution buffer (pH2.8) for 5min at room temperature. Eluting immuno-precipitated proteins were recovered by centrifugation and stored before use for kinetic analysis to determine interaction with orange II.

2.6. Surface Plasmon Resonance Interactions

Real-time biomolecular interactions between dentin matrix proteins and Orange II were performed using a BIAcore T100 system (GE Healthcare, Hatfield, UK). Human collagen type I (Sigma- Aldrich; dissolved at 1mg/ml in 0.5M acetic acid to obtain individual

collagen molecules), and immuno-precipitated decorin, biglycan, OPN and DSP (obtained as described above) were dissolved in sodium acetate buffer, pH4.5 (1 in 5) and immobilised onto a series S CM5 sensorchip (GE Healthcare) following the manufacturer's instructions. Briefly, the sensorchip was first treated with 70% (w/w) glycerol in water to normalize the flow cell surfaces and then activated by treatment with 0.05M N-hydroxysuccinimide and 0.2M N-ethlyl-N'-(dimethylaminopropyl)-carbodimide. Each protein was injected into a different flow cell, where the active ester groups on the surface on the chip surface spontaneously reacted with amines or other nucleophilic groups on the proteins molecules to form covalent links. Ethanolamine (1M) was injected over the flow cell surfaces after coupling, to deactivate any remaining esters. Flow cell 1 acted as a reference cell, exposed only to the amine coupling (control surface). An increasing series of Orange II dye concentrations (0–200 μg/mL) were prepared in HBS-EP buffer. Samples were injected over the immobilised proteins and control surfaces for 500s at a flow rate of 30μL/min. Sensorgrams were obtained for each Orange II dye concentration examined (n=4). Biacore T100 GxP Evaluation Software was used to evaluate the sensorgrams and calculate the mean equilibrium dissociation constant (K_D; M) based on the association rate constant (ka; $M^{-1}s^{-1}$]) and dissociation rate constant (kd; s^{-1}) data.

3. Results

3.1. Tooth Stain Development and Bleaching of Stained Tooth

The uptake of Orange II into the dentin samples was imaged using a stereoscopic microscope (Fig. 1A). Following conversion of images to a grayscale format, the percentage intensity of staining was calculated (Fig. 1B). Demineralised dentin samples demonstrated the greatest increase in orange II staining capacity. Intact dentin samples adsorbed less stain

compared with the demineralised samples ($p<0.001$ for all time points analysed). Intact dentine samples, however, adsorbed more stain compared to the deproteinized samples ($p<0.001$ for all time points analysed).

Dentin slices that had been stained for 168h (7 days) were subsequently treated with either 10% or 30% H_2O_2 for up to 48h (Fig. 2A) and the percentage grayscale as a measure of staining intensity was similarly calculated (Fig. 2B). Deproteinized samples lost their orange staining completely after 6h following treatment with either 10% or 30% H_2O_2 treatment. Loss of stain was slower for the demineralised samples, with significantly higher levels of orange II present in these samples at 3 and 6 h compared with deproteinized samples ($p<0.001$). For demineralised samples, complete loss of stain was only evident after 6h following 30% H_2O_2 treatment and 24h after 10% H_2O_2 treatment. The intact control dentin samples retained the orange II stain the longest, and had failed to return to pre-stained greyscale intensity values after 48h treatment with either 10% or 30% H_2O_2.

Following destaining with H_2O_2 for 48h, deproteinized (Fig. 3A) and demineralised samples (Fig. 3B) were then re-stained with Orange II dye for 168 h (7 days). Both tissue preparations indicated a significant reduction in the secondary Orange II uptake compared with the equivalent sample following 168h initial staining ($p<0.001$).

3.2. Effect of H_2O_2 on Collagen Type I

The effect of H_2O_2 on collagen fibrils was examined by SDS PAGE where the control untreated sample of collagen demonstrates the electrophoretic separation of the two á1 and the one á2 chains contained within the triple helix of the collagen molecules (Fig. 4). Samples were exposed to H_2O_2 at pH 3 and H_2O_2 adjusted to pH 8 to reduce the effects of an acidic environment. Following treatment with 3% and 6% H_2O_2 up to 24h no degradation of

collagen type I was observed at either pH (Fig. 4A, B and C). Treatment with 12% and 30% H_2O_2, at both pH, resulted in degradation of both of the collagen á chains, with increased presence of small molecular weight degradation products evident. Compared with H_2O_2 at pH 8, H_2O_2 treatment at pH 3 caused increased degradation of collagen type I at 1, 12 and 24h, for both 12% and 30% H_2O_2 concentrations (figures 4.A, B and C).

3.3. Kinetic evaluation of binding of dentin proteins with Orange II

Collagen type I, biglycan, decorin, OPN and DSP was immobilised to a S CM5 sensorchip at 8300RU, 351RU, 1500RU, 1014RU, 259RU, respectively, indicating good binding of the protein to the sensor chip. Orange II was passed over the immobilised protein and sensorgrams were obtained over an increasing concentration of Orange II (Fig. 5). For collagen type I, decorin and biglycan response units indicating interaction between the protein and the orange II increased with increasing concentration of the stain applied. From these sensograms mean equilibrium dissociation constant, K_D, was calculated, representing the ratio of the rate of dissociation of the dye from the protein (k_{off}; measured following inject stop of dye), / the rate of association of the dye with the protein (k_{on}; measured during injection of dye), providing an indication of the affinity of the dye for the respective protein. Collagen type I demonstrated a moderate affinity for Orange II with mean equilibrium dissociation constants (K_D) of 46 ìM. K_D for decorin and biglycan were calculated to be ~1000 ìM and ~1900 ìM respectively, indicating weak affinity for the Orange II. OPN and DSP bound poorly to Orange II, with any interaction too weak to accurately measure (K_D = > 2000 ìM).

4. Discussion

This study has investigated the interaction of Orange II with the dentin matrix, and been successful in providing an *in vitro* model to give greater insight and understanding of the internalisation of dietary stains within the dentine matrix. This discussion will expand on how the results lead us to hypothesise a major role for chromagen-binding sites on collagen type I in the adsorption process.

Within this study the uptake of Orange II was assessed on human dentin that had been prepared to a standardised thickness of 100μm to enable image analysis over a defined area for the quantification of dye uptake through a defined volume of tissue. The protocol was adapted from previous detailed studies that have compared the uptake of a range of potential chromophores including Rhodamine B, Orange II, Fe(III) phthalocyanine, and tea and which identified Orange II as idea for ease of measurement for tooth discolouration following uptake and subsequent total loss of the colour following treatment with hydrogen peroxide [24]. Our own results indicate a good ability of dentin sections to uptake the Orange II. However, following deproteinisation of the dentin sample with sodium hydroxide, staining was significantly reduced compared with the intact control. Sodium hydroxide has a well-established ability to extract proteins from mineralized matrices [26,27], which likely includes the non-collagenous components and potential for small soluble collagen molecules. Since samples remained relatively intact after treatment with sodium hydroxide, fibrillar collagen, representing the bulk of the matrix, is likely to still remain, although collagen fibrils have been shown to swell in sodium hydroxide [28], thus blocking potential chromagen binding sites. Demineralisation of dentin sections with EDTA increase staining with Orange II compare to the intact control, presumably due to the removal of hydroxyapatite crystals unmasking the protein-chromogen binding sites. As a consequence, removal of the stain following treatment with 10% H_2O_2 took longer for the highly stained demineralized sections (24h), whist deproteinized sections were beached within 6 h. The decreased staining of the

intact control group compared to the demineralised dentin samples, is likely due to reduced accessibility of the stain to the protein in the tooth structure. For a similar reason, these intact dentin samples required more time to completely bleach the chromagen with H_2O_2 treatment.

The strength of binding of the chromogen with the protein component is very pertinent when considering the permanency of the stain. Within this study, real-time kinetic analyses were performed making an assumption that although multiple sites are available on the protein molecules for binding of orange II, binding sites are independent of one another. The mean equilibrium dissociation constant, KD, was provided for collagen type I, decorin and biglycan, representing the molar concentration of the ligand (orange II) concentration at which half the binding sites are occupied at a steady state equilibrium. K_D values for dentin sialoprotein and osteopontin were >2000μM indicating that this steady state equilibrium was not achievable due to very poor attraction and interaction of the orange II with binding sites. However, while K_D values for decorin and biglycan were obtained, these were in the high molar concentrations, suggesting a weak affinity for the orange II. The mean equilibrium dissociation constant, K_D, for collagen type I was measured to be 46μM, suggesting a mildly moderate binding affinity for the orange II. Orange II dye is characterized by the presence of multiple phenol units, which are common with most tannin containing products such as wine, which stain teeth [24]. Previous studies investigating the interaction of phenolic polymers within tannins have notably demonstrated their selective interaction with proline-rich proteins such as collagen type I [29] and salivary proline rich proteins [30], which would support our observations for indicating a prominent role for dentine collagen type I in the adsorption of dietary stains. These previous studies have suggested that association is brought about through the hydrophobic stacking of the polyphenol rings against the *pro*-S face of the proline [30], which is then in turn predicted to be stabilised by hydrogen bonding between the phenolic hydroxyl and carbonyl oxygens within the peptide bond adjacent to the phenol

functional grouping on the protein chain [29]. The presence of proline results in a more extended structure, apparent in collagen type I, for hydrogen bond stabilisation, where globular proteins, existing as random coils, have been shown to bind less to polyphenolic tannins [29]. This observation may also aid in the further explanation of the above results assessing the reduced staining potential of deproteinized dentin samples. Whilst collagen was not fully extracted from deproteinized samples, the swelling of the collagen fibrils in the presence of sodium hydroxide may have blocked the penetration of the orange II to proline residues located in the interior regions of the collagen fibrils. The reconciliation of these results leads to a tempting hypothesis that dietary stains are attracted to and subsequently become incorporated within the collagen fibrils. Here, they are not necessarily strongly bound to the collagen fibrils but, following this initial hydrophobic attraction with proline groups and stabilisation with the adjacent peptide bond, dietary chromogens become entrapped in the dense mineralised collagenous matrix.

The underlying mechanism by which H_2O_2 results in bleaching of the chromogen is complex. In solution H_2O_2 decomposes to form hydrogen ions (H^+) and perhydroxyl ions (HOO^-) resulting in a weak acidic solution [18,19]. The perhydroxl ion interacts with further H_2O_2 to produce highly reactive oxygen species such as hydroxyl radicals perhydroxyl radicals. As strong oxidising bleaching agents, the free radicals are able to break chemical bonds within the phenyl structure of the chromogen. Raising the pH has been shown to increases the production of perhydroxyl ions which has been attributed to increasing its effectiveness as a tooth whitening product [18,19]. In addition through the Fenton reaction, H_2O_2 can react with exogenous ferrous ions (Fe^{2+}) to produce hydroxyl radicals. However, the activity of free radicals is indiscriminate resulting in the potential degradation of protein components or amino acid functional groups [20]. Against this background, it is of note that within the current study the subsequent ability to re-stain the H_2O_2 bleached deproteinized and

demineralized dentin samples over a 7 day period was reduced. *In vitro* studies performed herein, also indicate the ability of 10% and 30% H_2O_2 to depolymerize collagen fibrils, which would lead to the loss of the proline binding sites attracting the orange II stain. In addition, whilst depolymerization may not be evident, free radicals may also lead to the loss of functional groups of the amino acids which would reduce the stabilizing effect of the adjacent peptide bond [29]. Although not confirmed within this present study, previous studies have shown that proline, leucine, tyrosine and phenylalanine are particularly susceptible to degradation by free radicals [20]. This data suggests that controlled H_2O_2 treatment could help to reduce the uptake of external staining, although it is acknowledged that continuous exposure to H_2O_2 can lead to tooth erosion and tooth sensitivity. Of note, adjusting the pH of the H_2O_2 to pH 8 reduced the degradation of the collagen molecules, suggesting that collagen depolymerisation and bleaching of the phenyl structures in chromogens act via different mechanisms.

5. Conclusion

The results of this study suggest a major role for the protein constituents of teeth, particularly the proline-rich collagen type I, in attracting and moderately stabilising the interaction of dietary chromogenic substances with dentin matrix. Additionally, we have demonstrated how H_2O_2 can result in tooth whitening by bleaching of the stain, but also able to reduce re-staining, presumably due to the loss of these chromagen binding functional groups and peptide bonds on the collagen molecules which are known to be susceptible to degradation by H_2O_2 [20]. Within this study we investigated the effect of 30% and 10% H_2O_2, as a representation of H_2O_2 concentrations used in products used in dental clinics and over-the counter formulations. It is worth noting that numerous studies have investigated the effect of these concentrations of H_2O_2 on the mechanical properties (microhardness testing) and

ultrastuctural surface morphology (scanning electron microscopy, quantitiative profilometry) and gross chemical composition (X-ray diffraction, Raman spectroscopy) of dentine and found little effect of H_2O_2 containing products, even at the highest concentrations and following prolonged repeated exposure up to 7 days (review by [31]). This would suggest that although H_2O_2 may bring about subtle chemical changes reducing the attraction of the chromagen, non-excessive use of tooth whitening products may not be overly detrimental to the integrity of the dentine matrix and may contribute to reducing the re-staining of teeth.

Competing interests

The authors declare no financial and non-financial competing interests.

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Figure legends

Fig. 1: Comparison of colour changes between demineralized, deproteinized and intact dentin samples after staining with Orange I sodium salt for 1, 6, 24, 48, 168h and then viewed using a stereoscopic microscope (A). Using image analysis the intensity of the stain, was converted into a greyscale (B). Orange II adsorbed by the demineralized samples was significantly higher ($p<0.001$) compared with deproteinized samples at all-time points. After only 1 h, the intensity of Orange II dye into the demineralized samples was significantly higher compared with pre-stain samples ($p<0.001$); deproteinized samples took up to 24h for Orange II dye to become significantly higher than pre-stain samples. The intact control group at all-time points had less intensity compared with demineralized group and higher intensity compared to deproteinized group.

Fig. 2: The effect of H_2O_2 treatment (10% and 30% concentrations) on the discolouration of dentin slices stained with Orange II dye (A). Staining intensity was calculated using image analysis and is also presented (B). Stain was completely lost from deproteinized dentin samples after 3h using 30% H_2O_2 and by 6 h using 10% H_2O_2, although differences in staining intensity at 3h was determined not to be significant. Demineralized dentin samples took 24h to be completely bleached and there were no significant differences in bleaching efficacy between 10% and 30% H_2O_2 treatments. Significant differences between deproteinized and demineralized samples treated with both 10% and 30% H_2O_2 concentrations were identified at all time points examined up to 24h. Dentin samples in the intact control group, indicated that complete bleaching had not been achieved after 48h. Measurement of the grayscale intensity of Orange II showed significant differences between the intact control group compared to both demineralized and deproteinized groups.

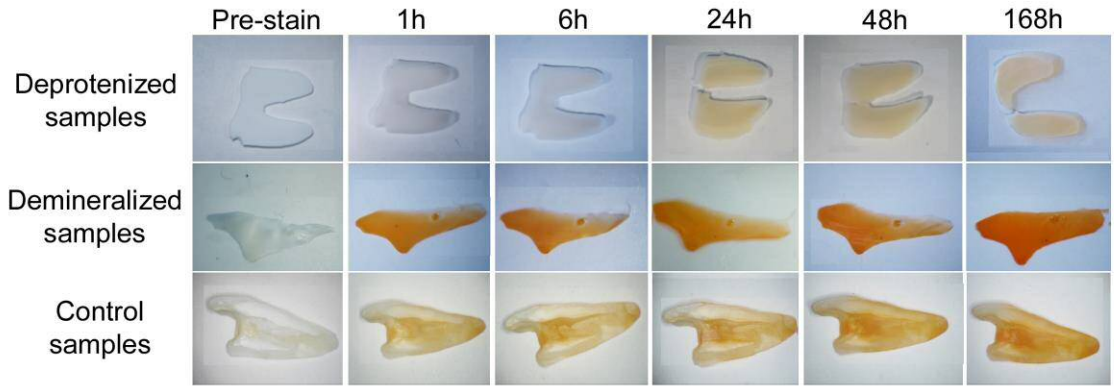
Fig. 3: The effect of Orange II staining before and after H_2O_2 treatment for 48h. Using either 10% or 30% H_2O_2 concentrations, deproteinized samples (A) and demineralized samples (B), showed reduced secondary Orange II uptake. The intensity of Orange II measured following analysis of grayscale images, before and after H_2O_2 treatment, showed significant differences ($p<0.001$) in all demineralized samples, and in deproteinized samples after 168h.

Fig. 4: The effect of H_2O_2 treatment on collagen type I protein examined following separation of collagen á-chains by SDS PAGE and silver staining. In the presence of 6% H_2O_2 there was no degradation of collagen type I even following 24h treatment (C). Compared with H_2O_2 at pH 8, H_2O_2 treatment at pH 3 caused increased degradation of collagen type I at 1, 12 and 24h, in the presence of both 12% and 30% concentrations (A, B, C).

Fig. 5: Representative sensograms for Orange II dye interacting with Collagen (A), Decorin (B), Biglycan (C), Dentin sialoprotein (D) and Osteopontin (E). Orange II was injected over the immobilised proteins at different concentrations over the time period indicated between the two arrows (injection on and injection off). From these profiles, BiaCore software calculated the Equilibrium Dissociation constant (K_D) which is shown for each protein. Values obtained indicate weak binding of orange II for the proteins decorin and biglycan. Binding with dentine sialoprotein and osteopontin was so weak that K_D values could not be calculated. Moderate binding was observed between Orange II and collagen type I.

Figure 1

(A)


Pre-stain
1h
6h
24h
48h
168h
Deprotenized samples
Demineralized samples
Control samples

(B)

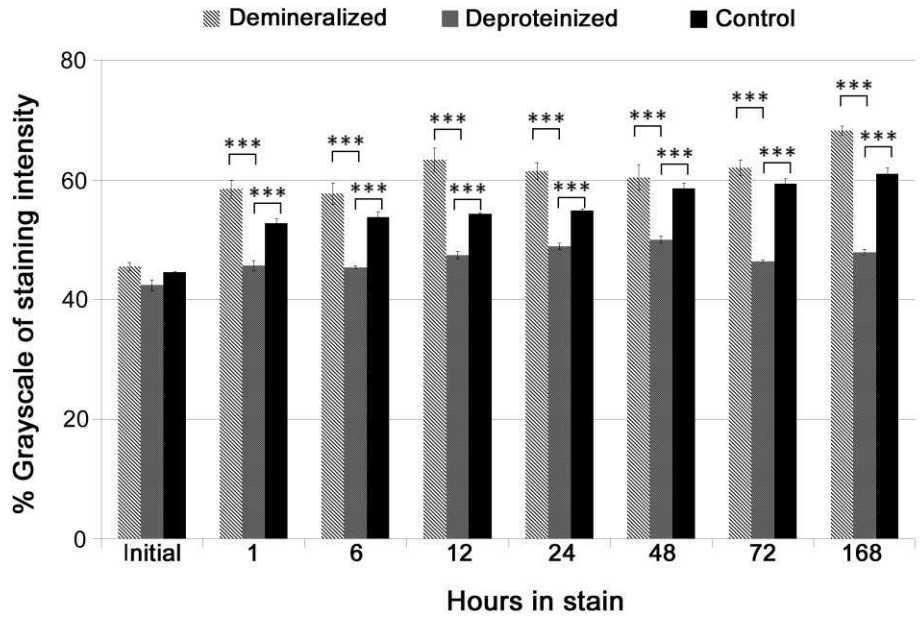
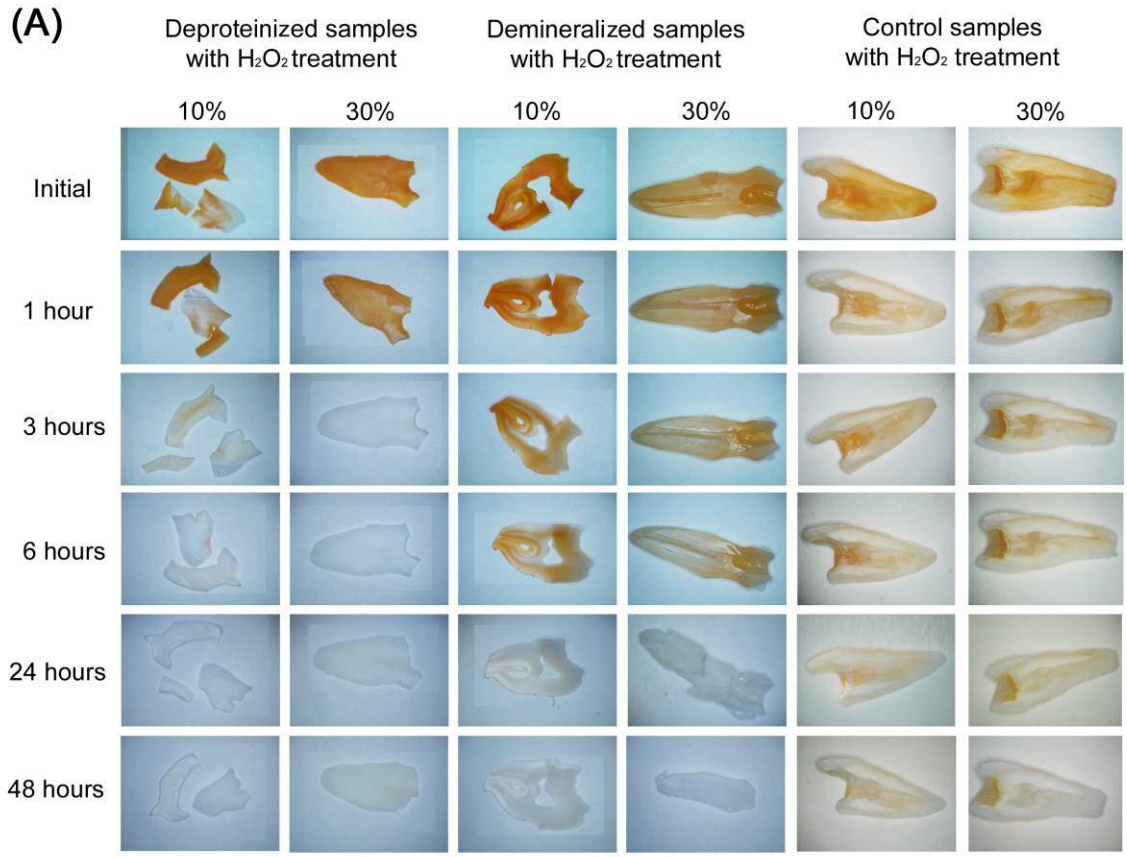

Demineralized
Deproteinized
Control
% Grayscale of staining intensity
0
20
40
60
80
Initial
1
6
12
24
48
72
168
Hours in stain
***

Figure 2


(A)
Deproteinized samples with H_2O_2 treatment
Demineralized samples with H_2O_2 treatment
Control samples with H_2O_2 treatment
10%
30%
10%
30%
10%
30%
Initial
1 hour
3 hours
6 hours
24 hours
48 hours

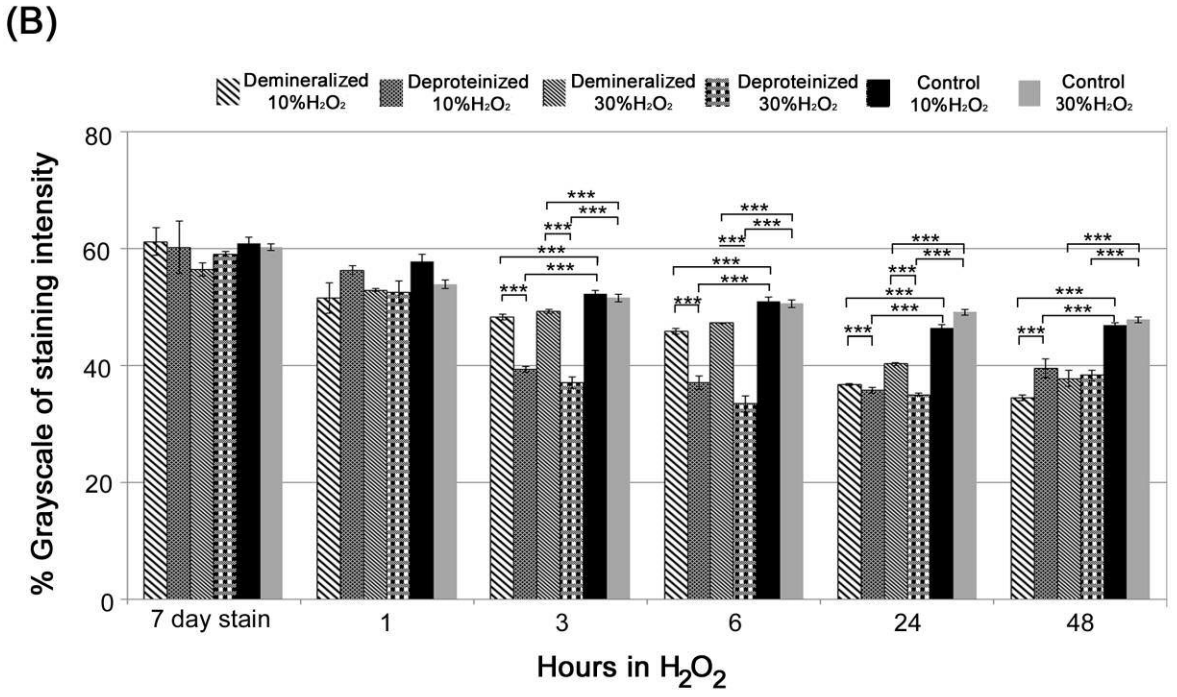

(B)
Demineralized 10%H_2O_2
Deproteinized 10%H_2O_2
Demineralized 30%H_2O_2
Deproteinized 30%H_2O_2
Control 10%H_2O_2
Control 30%H_2O_2
% Grayscale of staining intensity
0
20
40
60
80
7 day stain
1
3
6
24
48
Hours in H_2O_2

Figure 3

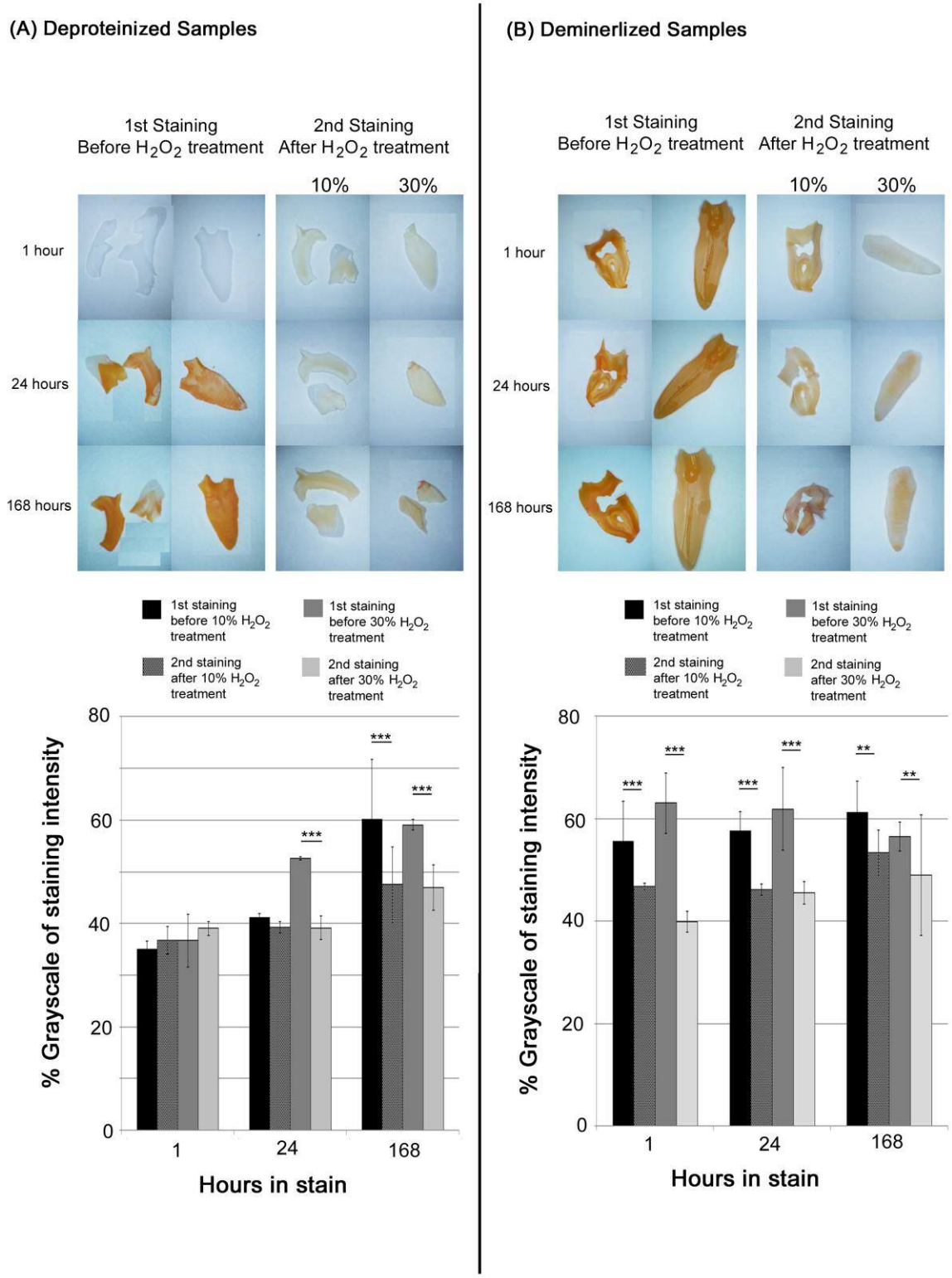
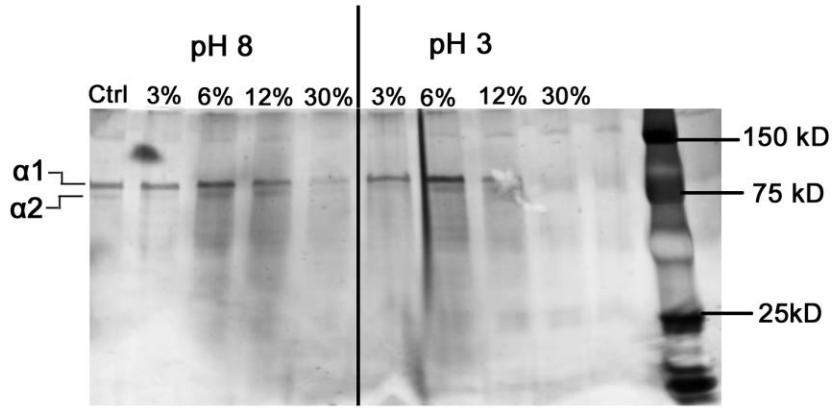
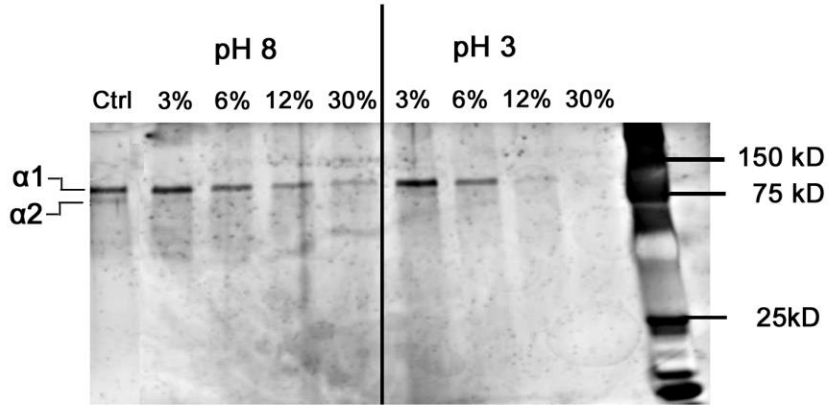

(A) Deproteinized Samples
1st Staining
Before H_2O_2 treatment
2nd Staining
After H_2O_2 treatment
10%
30%
1 hour
24 hours
168 hours
1st staining before 10% H_2O_2 treatment
1st staining before 30% H_2O_2 treatment
2nd staining after 10% H_2O_2 treatment
2nd staining after 30% H_2O_2 treatment
% Grayscale of staining intensity
Hours in stain
(B) Deminerlized Samples
1st Staining
Before H_2O_2 treatment
2nd Staining
After H_2O_2 treatment
10%
30%
1 hour
24 hours
168 hours
1st staining before 10% H_2O_2 treatment
1st staining before 30% H_2O_2 treatment
2nd staining after 10% H_2O_2 treatment
2nd staining after 30% H_2O_2 treatment
% Grayscale of staining intensity
Hours in stain

Figure 4

(A) Collagen type I after 1h treatment with H_2O_2



(B) Collagen type I after 12h treatment with H_2O_2



(C) Collagen type I after 24h treatment with H_2O_2

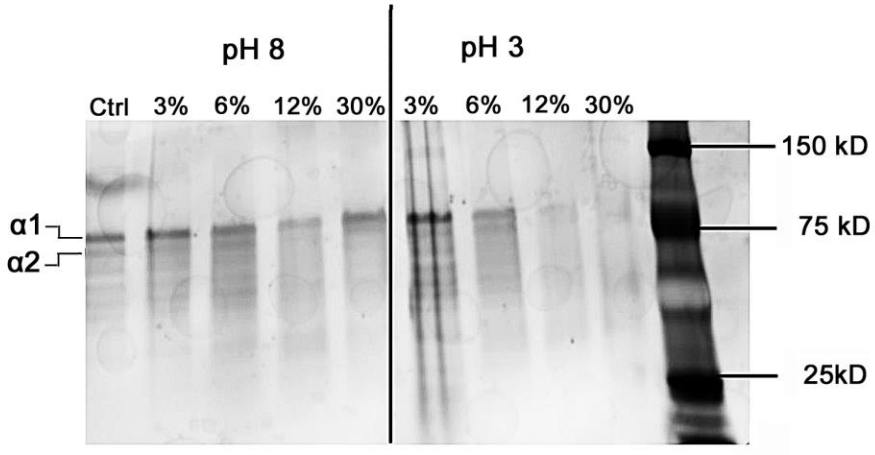
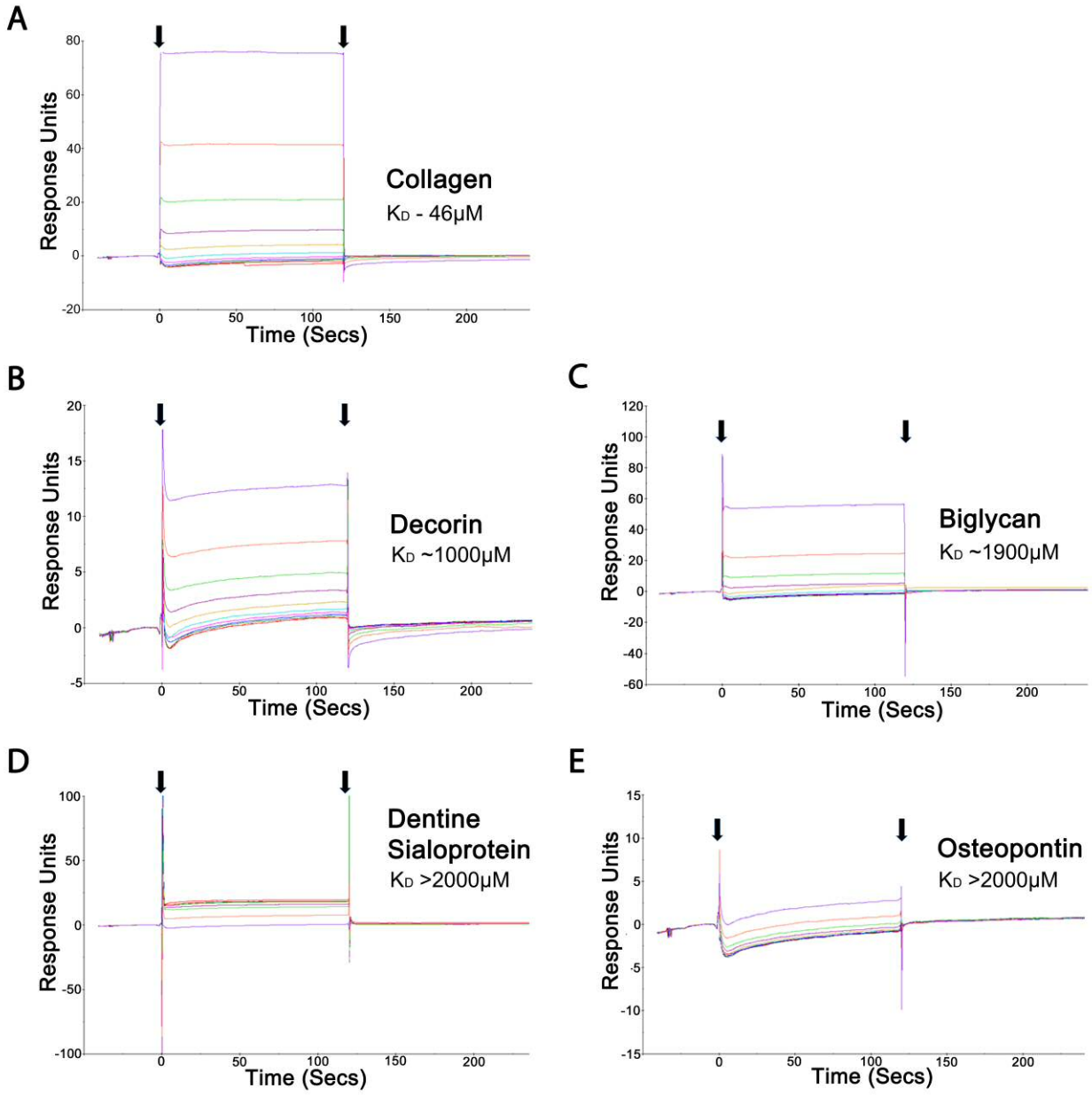


Figure 5


A
Response Units
Time (Secs)
Collagen
KD - 46μM
B
Response Units
Time (Secs)
Decorin
KD ~1000μM
C
Response Units
Time (Secs)
Biglycan
KD ~1900μM
D
Response Units
Time (Secs)
Dentine
Sialoprotein
KD >2000μM
E
Response Units
Time (Secs)
Osteopontin
KD >2000μM